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List of communicated papers

1) SFC-MS/MS for identification and estimation of the Ethambutol in its dosage form and human urine samples. (Journal of Chromatography B)
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Analysis and impurity identification in pharmaceuticals

Abstract: Impurity is not a much-liked word by pharmaceutical and industry people, because they are concerned about quality. Here we discuss various impurities that might be present in API formulations. To fulfill our purpose we have compiled a variety of regulatory authorities’ guidelines (i.e., ICH, WHO, and pharmacopoeias), which serve in endlessly regulating the impurities by various means. As the impurity present in a drug can affect its quality and thus its efficiency, it is therefore crucial to know about impurities. The current article reveals the different terms, regulatory control, and basic techniques (e.g., HPLC, LC-MS, TLC) that will help novices to understand, identify, and quantitatively estimate impurities and that have the advantage of profiling. This article primarily focuses on identification and control of various impurities (i.e., organic, inorganic, and genotoxic). For any of the substances, quality is the prime objective. Because impurities can alter quality, understanding the various impurities will help in producing quality products.

Keywords: analytical methods; genotoxic impurity; inorganic impurity; organic impurity; regulatory requirement in impurity profile.

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Introduction

The pharmaceutical world is dedicated to quality. Speaking from the customer’s perspective, quality means pleasant appearance with good packaging. But in the case of pharmaceutical industries, quality means providing drug standards conforming to a variety of conditions and making profit from them. So, they should be aware of the various types of impurities and their regulation and control, which infer quality. Therefore, in this paper we have tried to summarize different types of impurities, along with their effects and limitations as given by the International Conference on Harmonization (ICH). ICH has given guidelines [ICH Q-3B (R2) 2006] for impurity in a drug, and according to ICH, it is a chemical entity, which is not defined as a drug per the Drugs and Cosmetic act and which has an impact on the purity of the active pharmaceutical ingredient or drug substance.

Every pharmaceutical manufacturer defines impurity in its own words, making it difficult to find an exact definition of impurity. In the pharma world, impurity can be identified by various terms that we will see later. Drug substances or drug products are prepared with various solvents. Remaining solvents or residual solvents that might be present in the final product often are cited as organic volatile impurities (OVI) (ICH Q-3C [R4] 2009), and the impurities associated with the inactive pharmaceutical ingredients used in formulation or as additives or adjuvants are rarely mentioned.

Bulk pharmaceutical chemicals (BPCs), can be obtained or synthesized from multiple sources and, therefore, it is very important that impurities in BCPs be carefully monitored and controlled. Recently British pharmacopoeia (BP), United State Pharmacopoeia (USP), and Indian pharmacopoeia (IP) started incorporating allowable limits of impurities present in drug substances or drug products (Kovaleski et al. 2007, Gad 2008). This article thoroughly reviews different impurities found in the pharmaceuticals by methods for isolation, extraction, and identity of possible impurities.

Impurity should be defined as identified impurity – an impurity available with information about the structural characterization, and unidentified impurity – an impurity that can be identified only with qualitative analytical values (e.g., peak area, retention time, etc.), for which structural information is not yet available.

Impurities present in new drug substances used in clinical and safety trials are covered under two aspects [ICH Q-3A (R2) 2006]. Chemistry aspects classify and identify impurities, generate the report for different impurities,
list various impurities present in any substances, and give a brief discussion of analytical procedures for impurity detection. Safety aspects include those impurities that are present at a considerably lower amount or not present at all in a discovery of new drug substance.

Commonly used impurity terms

A number of terms have been commonly used to describe an impurity or impurities (Francis et al. 1984, McNaught and Wilkinson 1997):

- **Intermediate**
- **Penultimate intermediate**
- **By-product**
- **Transformation product**
- **Interaction product**
- **Related product**
- **Degradation product**
- **Foreign substance**
- **Toxic impurity**
- **Concomitant component**
- **Ordinary impurity**
- **Organic volatile impurity (OVI)**

**Intermediate**

The compounds formed in the process of synthesis for the desired product are called intermediates or reaction intermediates. They are defined as products that have undergone a partial processing and are used as raw material in a successive productive step.

**Penultimate intermediate**

As the name suggests, this is the compound found in the synthesis chain before the production of the desired compound. Sometimes confusion arises when the desired material is a salt of a free base or acid. In our opinion, it is inappropriate to label the free base or acid as the penultimate intermediate if the drug substance is a salt.

**By-product**

The unintentional compounds that arise during the reaction are commonly called by-products. Not all by-products can be quantified easily; hence, they present a thorny problem to the analytical chemist. A by-product can be useful and marketable or it can be considered waste.

**Transformation product**

This relates to an expected and non-expected product that may be formed in the reaction. Transformation products are very similar to by-products, except the term tends to connote that more is known about the reaction products than transformation products.

**Interaction product**

This term is slightly more comprehensive and more difficult to evaluate than by-products and transformation products in that it considers interactions occurring among various chemicals involved in reaction.

**Related product**

As mentioned, impurity is a word that is not well liked. So a related product actually is similar to an impurity, but active pharmaceuticals use the term related products instead, thus playing down the negativity frequently attached to the term impurity. These products can have similar chemical structure and might have standardized biological activity; however, this by itself does not provide any guarantee of effect.

**Degradation product**

The compounds produced due to decomposition of the material of interest or active ingredients often are referred to as degradation products.

**Foreign substance**

This is the material that may be present due to contamination or adulteration, not as outcomes of synthesis.

**Toxic impurity**

Toxic impurities might affect the biological activity, even at very low concentrations. They require identification by qualitative or quantitative means.
Concomitant component

Bulk pharmaceutical chemicals may contain concomitant components, which are geometric and optical isomers and antibiotics that are mixtures.

Ordinary impurity

An impurity having enough potency to have biological activity – even at trace level – is called an ordinary impurity.

Organic volatile impurity

A solvent that may remain in the drug substance should be considered as an organic volatile impurity (OVI).

Classification of impurities

- Organic (process and drug related)
- Inorganic
- Residual solvents
- Polymorphic
- Enantiomeric.

Organic impurities

Organic impurities come into existence during the synthesis of the active and inactive materials. They may occur during manufacturing or during storage of the materials. These impurities can be deduced from degradation reactions and ongoing synthesis in active pharmaceutical entities and drug products. Impurities generated during the synthetic process are intermediates, by-products, and reagents, as well as ligands and catalysts used in the chemical synthesis (Ahuja 1998, Qiu and Narwood 2007).

Starting materials and intermediates

These are the chemical compositions used to synthesize the desired constituent of a drug substance molecule. Starting materials and intermediates that are not reacted in the reaction, especially when the synthesis is about to complete, will remain in the final product as impurities (Muehlen 1992, Gorog 2000, Gavin and Olsen 2006). One such example is 4-aminophenol, a starting material for synthesis of paracetamol bulk drug, which might be present in final product as an impurity having a toxic effect on the liver.

According to Dir. 2001/83/EC (EMEA 2012), for biological medicinal products, “Starting materials means any substance prevailed from the human or plant or microorganisms or any alteration to the biological origins by means biotechnological cell constructs which will have tendency to formed drug product.” So measures for controlling sourcing of starting materials or intermediates must be strong.

An intermediate is a substance that is produced in the reaction vessel from the starting materials and which might undergo further chemical modification to provide the final product.

By-product

As mentioned earlier, the desired product is commonly called the main product, and product that is unwanted but might be useful is known as a by-product.

Degradation product

Degradation products are the compounds formed due to chemical changes in drug products during storage. Degradants may form because of chemical interactions with other compounds or due to contaminants present in the drug substances.

In certain cases, physical degradation occurs for a variety of reasons: change in the polymorphic state of the molecule, aggregation of proteinaceous material due to heat or residual solvents, absorption of water, loss of water, and others. A degradation product can be determined by short- and long-term stability studies per ICH, for example, in treatment for common cold formulations containing acetaminophen, phenylephrine hydrochloride, and chlorpheniramine maleate. Degradation products for these formulations were isolated and found to be an addition compound of phenylephrine and maleic acid (Wong et al. 2006). The definition of degradation product in accordance with the ICH guideline is “any chemical change occurring due to overreaction or over heating or changing in condition of solution, i.e., change in pH, exposure to light, etc. or reaction of final product with container or closure or excipients used in making product” [Gorog 2003, ICH Q-3A (R2) 2006].
Reagents, ligands, and catalysts

Reagents, ligands, and catalysts are seldom present in the final products (Ahuja 1998, Roy 2002). For the synthesis of the drug substance or any excipient catalysts, chemical reagents and ligands are used that can be conveyed to the concluding products as impurities in minute levels. For example, carbonic acid chloromethyl tetrahydro-pyran-4-yl ester (CCMTHP) (Gorog 2003), is an alkylating agent that was observed as an impurity in the synthesis of a β-lactam drug substance.

Products of overreaction

Products of overreaction form when reactions for the synthesis are not selective as much as necessary, so nonselective interaction at an undesired site will produce an incorrect compound. For example, the last step for the synthesis of nanodralone decanoate is the decanoylation of the 17-OH group. Enol compound 3, 17β-dihydroxyestra-3,5-diene disdecanoate was formed because of overreaction at the 4ene-3 oxo group site (Gorog 2000, 2003).

Contamination by organic impurities

Contamination with organic impurities is not related to a drug but might unknowingly be present in the drug. For example, for drug substances derived from plants, herbicides used to protect plants may be present, such as diquat and glyphosate, or pesticides such as carbofuran and endrin sprayed into the environment (Bauer et al. 2001).

Inorganic impurities

Inorganic impurities include filter aids, color removing agents such as charcoal, reaction rate modifiers (catalysts), ligands, and heavy metals. One example would be a catalyst used in a substitution reaction during the synthesis of the API or raw materials. Inorganic impurities might have toxic effects, so they should be removed or controlled to a minimum level. Batch-to-batch variation in impurity levels suggests that the manufacturing or synthesis process of the drug product is not controlled (Roy 2002, Basak et al. 2007, Hulse et al. 2008, ICH Q-3D 2009).

Inorganic impurities normally known and identified are as follows.

Contamination by inorganic impurities

These are unforeseen impurities found in final product. Contaminant impurities detected in drugs have been controlled in many ways. For example, previously used glass vessels for reaction are now replaced with acid/alkali resisted glass (Bauer et al. 2001). So, impurities that might be present due to leaching from glass vessel is minimized to safer levels.

Reagents, ligands, and catalysts

Reagents, Ligands and Catalysts are well defined under organic impurity of this paper. However, catalysts used in decomposition of intermediates (iodide catalysts), and monodentate ligand such as chloride ions might remain in the final product as inorganic impurities.

Residual solvents

Residual solvents in pharmaceuticals are the volatile chemicals that are produced as a result of side reactions or used in the manufacturing of API or excipients, or in the formulation [ICH Q-3C (R4) 2009]. Theoretically they can be removed from the final product but practically they can not. Therefore, it may be a vital parameter in the process for making a drug product.

Polymorphic forms

Solid material that subsists in two or more forms or in a crystalline structure is said to be polymorphic. Some organic and inorganic compounds form different crystalline structures called polymorphs or polymorphic forms. The resulting change of intermolecular interactions gives rise to different pharmacokinetic properties of medical drugs, as well as to different properties of organic and inorganic materials. Therefore, the unambiguous identification and characterization of polymorphs is very important, especially from the economic point of view. In 2006 a new crystal form of maleic acid had arisen when solution of caffeine and maleic acid (2:1) in chloroform is set aside to evaporate slowly (Day et al. 2006).

Enantiomeric impurities

To determine purity of the chiral compound term enantiomeric excess (EE) is used. These impurities present in the
drug are due to change in the critical parameter of molecules during synthesis. The following equation is used to determine enantiomeric excess (EE):

$$EE = \frac{([R-S])}{([R]+[S])} \times 100$$

where R and S stand for the individual optical isomer in the mixture (and R+S=1).

These determinations are important particularly when we are talking about efficacy of the drug, because in the case of optical isomers of a drug only one isomer has therapeutic efficacy while the rest of them have either a toxic effect or have no effect at all (Armstrong et al. 1998, Roy 2002, Gorog 2003, Qiu and Narwood 2007).

### Control of impurities

According to theory, all impurities should be removed from the final product, but in practice, impurities cannot be entirely abolished from the final product. So, for a quality product, impurities should be kept within the limits. According to a study carried out for impurity, very low amount of impurities in the product should be allowed. However, in special cases, rather high quantities of impurities are permitted, for example, biotechnologically derived products that have biological activity.

Most of the bulk pharmaceutical chemicals (BPCs) are obtained from various sources. Therefore, it is crucial that impurities in BPCs be monitored and controlled very carefully.

Various controlling authorities for impurity (USP 1995, ICH Q-6A 1999, ICH Q-6B 1999) are mentioned in monographs and specifications about maximum tolerable limits.

### Control of organic impurity

Most often, reduction in quantity of by-products in the reaction can be carried out by tightly controlled reaction conditions at crucial steps of the reaction to preclude a new impurity or diverging level of impurity. Another approach to reduce the quantity of impurity in the final product is to use superior quality starting materials. Likewise, the use of high-grade solvents also imparts its effort to obviate the production of by-products or any unknown entity. The thresholds for allowable organic impurities are shown in Table 1.

### Control of degradation impurity

This particular impurity covers degradation products of active substance, including reaction products with excipient or container system [ICH Q-3A (R2) 2006, ICH Q-3B (R2) 2006]. Degradation products observed in stability studies performed at recommended storage conditions should be identified, qualified, and reported when the following thresholds exceeded (Table 2).

### Control of inorganic impurities

Oral/parenteral concentration limits (ppm) have been proposed for 14 metals in active substances or excipients: Pt, Pd, Ir, Rh, Ru, Os, Mo, V, Ni, Cr, Cu, Mn, Zn, and Fe. Metals are divided into three classes as follows, and limits have been summarized in Table 3.

#### Class 1: Metals of significant safety concern

Some metals are known or suspected human carcinogens, genotoxic, and sometimes nongenotoxic carcinogens or...
potential contributory agents which produce irreversible
toxicity, for example, neurotoxicity or teratogenicity. A
few of them produce significant but reversible toxicity,
such as Ir, Pd, Pt, Ru, Os, Mo, V, Cr, and Ni.

Class 2: Metals with low safety concern

Trace metals required for nutritional purposes can be
present in foodstuffs or as readily available supplements,
for example, Cu and Mn.

Class 3: Metals with minimal safety concern

Metals, omnipresent in the environment or plant and
animal kingdoms, as such have high tolerable toxic values
for humans. The nutritional intakes of ≥ 10 mg/day is rec-
commended. Examples are Fe and Zn.

Control of residual solvents

Various regulatory authorities have been concerned about
toxicity of the residual solvent in the pharmaceutical
world. At most, various pharmaceutical provide guidelines (USP 1990, BP 1996, EP 1997, EMEA 2009) for the
control of residual solvents and with different categories
in pharmaceuticals gives acceptance limits (Table 4). In
addition, for solvents that are used in pharmaceuticals,
there are only a few residual solvents that are controlled
(Hu and Liu 2011). So globally there is a need for a stand-
ard guideline to be established for the control of residual
solvents. Therefore, the harmonized guidelines for control
of residual solvents by ICH has been released.

For pharmaceutical production, organic solvents
invariably remain present in the processes. The pharma-
caceutical industry is one of the largest users of organic sol-
vants per amount of the final product (Slater et al. 2006,
Smith and Webb 2007, Katarzyna and Andrzej 2010). The
synthesis of an active or inactive pharmaceutical ingredi-
ent usually requires large amounts of solvent and some-
times during the drug product formulation process, as well
as during the formulation process methylene chloride,
is used as solvent in large amounts for coating process.
Residual solvents are placed in following classes based on
their toxic effects to human health.

Class 1 solvents: solvents to be avoided

Solvents in class 1, due to their known carcinogenicity
and hazardousness to environment should not be utilized
in the manufacturing of active and inactive materials, or
drug products. Even so, in any circumstances, if we can
avoid use of this class of solvents, they should be limited
in the final product as shown in Table 5.

Class 2 solvents: solvents to be limited

Solvents listed in Table 6 might be less toxic than class 1 sol-
vants, but because of their inherent toxicity they should be
limited as PDEs. This class is very much higher than class 1.

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### Table 3  Limits of inorganic impurities in oral and injectable (CPMP/SWP/QWP/4446/00).

<table>
<thead>
<tr>
<th>Classes of metals</th>
<th>Oral</th>
<th>Injectable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDE (μg/day)</td>
<td>Concentration (ppm)</td>
</tr>
<tr>
<td>Class 1A: Pt, Pd</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Class 1B: Ir, Rh, Ru, Os</td>
<td>100b</td>
<td>10b</td>
</tr>
<tr>
<td>Class 1C: Mo, Ni, Cr, V</td>
<td>300</td>
<td>30</td>
</tr>
<tr>
<td>Class 2: Cu, Mn</td>
<td>2500</td>
<td>250</td>
</tr>
<tr>
<td>Class 3: Fe, Zn</td>
<td>13,000</td>
<td>1300</td>
</tr>
</tbody>
</table>

*a Separate limits for inhalation exposure to Pt, Cr (VI) and Ni.
*b Subclass limit.
PDE, permitted daily exposure.

### Table 4  Limits of initially controlled residual solvents in pharmacopoeias.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Benzene</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Trichloroethene</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>–</td>
<td>50</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td>Pyridine</td>
<td>–</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Toluene</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5  Solvents in pharmaceutical products that should be avoided (ICH Q3-C).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration limit (ppm)</th>
<th>Concern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>2</td>
<td>Carcinogen</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>4</td>
<td>Toxic and environmental hazard</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>5</td>
<td>Toxic</td>
</tr>
<tr>
<td>1,1-Dichloroethene</td>
<td>8</td>
<td>Toxic</td>
</tr>
<tr>
<td>1,1,1-Trichloroethane</td>
<td>1500</td>
<td>Environmental hazard</td>
</tr>
</tbody>
</table>

Class 3 solvents: solvents with low toxic potential

Solvents in this class have low toxic potential to humans, as these solvents have PDEs of 50 mg or more per day.

Class 3 solvents which should be limited by GMP are as under (per ICH Q3C):

1-Butanol  Methyl acetate  Heptanes
Acetone  Isobutyl acetate
2-Butanol  3-Methyl-1-butanol
Anisole  Isopropyl acetate
Acetic acid
Methyl ethyl ketone
Butyl acetate
1-Pentanol  Methyl acetate  Ethanol
1-Propanol  Tert-Butylmethyl ether
Methyl isobutyl ketone
2-Methyl-1-propanol  Ethyl format
2-Propanol  Cumene
Pentane  Formic acid
Dimethyl sulfoxide

Other class: solvents for which no adequate toxicological data was found

This class lists additional solvents for which no adequate toxicological data available to generate a PDE. Some examples are (ICH Q3C 2009)

| Isooctane              | 1, 1-Dimethoxymethane  | Petroleum ether          |
| Methyl isopropyl ketone| 1, 1-Diethoxypropane   | Trifluoroacetic acid     |
| 2, 2-Dimethoxypropane  | Isopropyl ether         |                          |
| Methyltetrahydrofuran  | Trichloroacetic acid    |                          |

Control of genotoxic impurities

Existing ICH Q-3 guidelines do not provide acceptable toxicological limits of genotoxic impurities in active pharmaceuticals. Determination of genotoxic effects of impurities without any data is very difficult for assessing impurity. Most of the pharmaceutical and other concerned industries accept the approach of threshold of toxicological concern (TTC). This approach gives an acceptable risk value (a TTC value of 1.5 μg/day intake) for intake of genotoxic impurity for most pharmaceuticals.

Various classes of genotoxic impurities are as follows (McGovern and Jacobson-Kram 2006):

- Class 1: Impurities known to be genotoxic (mutagenic) and carcinogenic
- Class 2: Impurities known to be genotoxic (mutagenic) but with unknown carcinogenic potential
- Class 3: Alerting structure, unrelated to the parent structure and of unknown genotoxic (mutagenic) potential
- Class 4: Alerting structure, related to the parent API
- Class 5: No alerting structure or indication of genotoxic potential.

During clinical trials some data that signifies the allowable daily intake is summarized in Table 7 (EMEA 2006, McGovern and Jacobson-Kram 2006).
Isolation and characterization of impurities

A number of methods can be used for isolating and characterizing impurities. The application of any given method depends on the nature of the impurity, in other words, its structure, physical and chemical properties, and availability (the amount present in the original material from which it must be isolated). The following methods may be useful in this context:

- Extraction
- Chromatography
- Preparative separations.

Extraction

Extraction is one of the most useful methods for isolation of an impurity. For this the following methods can be helpful:

- Liquid/solid extraction
- Supercritical fluid extraction
- Liquid/liquid extraction or solvent extraction.

Normal phase SPE

The theory involved in normal phase SPE generally require mid- to nonpolar solvent mixtures (e.g., n-hexane, methylene dichloride, acetic acid, diethyl ether, etc.), a polar substrate (e.g., drug molecule, excipients, etc.) and a polar stationary phase. For the normal phase, various stationary phase materials are used. One of them is silica, which can be modified further with polar heads (e.g., Si-C4-CN, Si-C4-NH2, etc.). Other adsorbents used are florisil, alumina, etc.; the mechanism involved in retention of substrate in normal phase SPE is principally the interaction with a polar analyte functional group and polar heads in the stationary phase.

Reversed phase SPE

The mechanism involved in reversed phase SPE requires a polar mobile phase (e.g., methanol, ethanol, water, etc.) or a semi-polar solvent mixture and a nonpolar stationary phase. In the reverse phase SPE modified silica is used as the stationary phase, in other words alkyl- or aryl-bonded silicas (Si-C-18, Si-C-8, Si-C-4, and Si-C-Ph).

Ion exchange SPE

The main rationale of the ion exchange SPE is to separate oppositely charged ions in a solution. Different types of exchangers have been used to separate the charged moieties. Commercially available ion exchangers contain resinous parts having amine or quaternary ammonium groups or other ionic groups for the separation of anionic or cationic compounds. The retention mechanism for the analyte is at the exchanger surface for the diffusion of ions. This depends on the concentration of the solution and the degree of cross linking of ion exchangers.
Anion exchange SPE
Material used in anion exchange SPE for the stationary phase is having a positively charged group (e.g., an aliphatic quaternary amine group or amino group). Positively charged groups such as quaternary amines are strong bases that will draw anionic molecules into the solution and strongly attach to the exchanged group. As it strongly binds to the anionic group, it is termed a strong anion exchanger (SAX). Because of its strong binding capacity, it is generally used when recovery of anion is no longer required. However anions that can be displaced by another anion shall be eluted by changing the pH of the solution.

The stationary phase containing amino group, used in the normal phase SPE, can be used as a weak anion exchanger (WAX). The advantage of WAX utilization for separation of species is that we can isolate and recover strong as well as weak anions.

Cation exchange
The materials used for cation exchange are high molecular weight cross-linked polymers having carboxylic, phenolic, or aliphatic sulfonic acid groups. Among these groups sulfonic acid pulls in cationic species strongly present in solution and so is termed a strong cation exchanger (SCX). Moreover, materials containing a carboxylic or phenolic group that is a weak anion can be used as weak cation exchanger (WCX). By the use of WCX, strong and weak cations can be isolated and recovered easily.

Supercritical fluid extraction
In the field of supercritical fluid extraction (SFE) (Hedrick et al. 1992, Wai and Laintz 1994, Simpson 2000, McHugh and Krukonis 2008) various researchers proposed the use of supercritical carbon dioxide (CO₂) as an extractant for separating various components.

The procedure involved in SFE is very convenient for novices. A sample thimble is used to handle a sample through which supercritical fluid is pumped. The extraction of the soluble compounds is allowed to take place as the supercritical fluid passes into a collection trap through a restricting nozzle. After passing through the nozzle, it is recompressed by venting in the collection trap for future use. The material left behind in the collection trap is the product of the extraction. Characteristics of gases normally using SFE are given in Table 8.

### Table 8 Solvents for SFE.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Pressure (atm)</th>
<th>Temperature (°C)</th>
<th>Density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Pentane</td>
<td>33.6</td>
<td>196.6</td>
<td>0.232</td>
</tr>
<tr>
<td>CO₂</td>
<td>72.9</td>
<td>–</td>
<td>0.448</td>
</tr>
<tr>
<td>NH₃</td>
<td>111.3</td>
<td>132.3</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Liquid/liquid extraction or solvent extraction
In liquid-liquid extraction components are separated based on their solubility in two slightly miscible or completely immiscible solvents, where mass transfer occurs at the interface and components separate by their affinity to the solvents (Qiu and Narwood 2007, Aguilar and Cortina 2008).

Partition coefficient plays an important role in this extraction process, by which the amount of solute that is distributed between two immiscible solvents a and b can be easily found:

\[ K_d = \frac{C_a}{C_b} \]

where, \( K_d \) is the distribution coefficient or partition coefficient.

\( C_a \) is the concentration of component in solvent a.

\( C_b \) is the concentration of component in solvent b.

By the use of this technique the solution containing impurity can be concentrated and thus the impurity can be easily detected.

Chromatography
Most recently, organic drug substance impurities are measured using chromatographic procedures, as they give more accurate results. These procedures should involve a separation mode that allows for the resolution of impurities from the drug substance and a detection mode that allows for the accurate measurement of impurities.

Owing to the polar and nonvolatile nature of most compounds used as medicinal drugs, reversed-phase HPLC is the most common technique for monitoring the drug substance and its impurities. GC is also used, particularly for residual solvents, and capillary electrophoresis (CE) has been introduced in more recent times. Some older methods use thin-layer chromatography (TLC), but use of this methodology for the quantitative measurement of impurities is not common.
HPLC-MS or HPLC-NMR

The most common technique for monitoring impurities is HPLC with UV detection. Quantification of impurities is achieved by reference standards, when available, or by area percent or height percent relative to the parent compound (Lee and Kerns 1999, Kostiainen et al. 2003). Important application for impurity identification with HPLC is by the use of MS as detector.

Recently, HPLC-MS has become the popular technique for structural elucidation and confirmation of impurities. During the synthesis it is necessary to identify the various types of impurities for maintaining the quality of the product. Because of its selectivity, sensitivity, and compatibility with LC, LC-MS and LC-NMR have become absolutely necessary analytical techniques for the analysis of impurities present in various drugs and drug products and have become the first choice method. As it provides some structural information about fragments, empirical formula, and molecular weight, it has become a popular and advantageous method for the impurity analysis.

Coupling of LC and NMR (Treiber 1987, Albert 2002) has recently attracted research because of reduction in tedious preparative steps and substantially acquires higher efficiency and precision when handling complex mixtures.

Thin-layer chromatography (TLC)

For isolation and purification of compounds, TLC has gained importance because of its simplicity and utility. No major equipment is required, and the method of development is relatively easy (Sherma and Fried 1991, Ahuja 2003, Smith and Webb 2007). The primary limitation is the small number of theoretical plates that are obtained with this method as compared to GC or HPLC.

Detection frequently is performed visually or by UV (e.g., 366 nm). The fluorescence-quenching substances absorbing UV light in the short-wavelength region also can be detected if the layer is impregnated with a fluorescent substance. Iodine vapors can help detect most organic substances. A number of techniques can be used to recover the sample from the plate. The most simple and convenient method for obtaining the desired material is scraping the sorbent from the adsorbent site and shifting it to an extraction vessel, where different solvents are used for extraction of a compound.

Capillary electrophoresis (CE)

CE is not used much for impurity identification, but it offers the advantage that CE procedures can be employed when HPLC procedures have failed to measure the impurities adequately. CE is particularly important for the separation of chiral compounds that have closely related structures.

References


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SFC–MS/MS for identification and simultaneous estimation of the isoniazid and pyrazinamide in its dosage form

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A B S T R A C T

An ecofriendly and sensitive SFC–MS/MS method (using TurbolonSpray probes) has been developed to measure isoniazid (INH) and pyrazinamide (PYZ) in fixed dosage combination (FDC) by dissolving in dichloromethane:methanol:formic acid (50:50:0.1 v/v/v). Supercritical carbon dioxide (SC-CO₂) is used as mobile phase at a flow rate of 2 ml/min and modifier used is dichloromethane:methanol:formic acid (50:50:0.1 v/v/v) at a flow rate of 0.3 ml/min. High penetration power, low viscosity, negligible toxicity makes the obvious choice for environment friendly mobile phase. The separation of INH and PYZ was achieved in less than 5 min using a C18 reverse-phase fused-core column (Inertsil ODS-5 µm C18, 150 mm × 4.6 mm). The method was validated as per international standards in terms of selectivity, linearity, precision and recovery. The method was found to be linear and % recovery was found to be 98.89–100.33% and 99.27–100.6% for INH and PYZ, respectively. Lower limits of detection and quantification could be achieved for INH was 11.87 ng/ml and 35.97 ng/ml, respectively and for PYZ was 35.42 ng/ml and 107.36 ng/ml, respectively. The proposed method was applied to the marketed formulation of different manufacturers.

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1. Introduction

The concept of “Green Separation” was taken for the environmentally consented chemical process [1]. In this process, we are limiting the use of hazardous chemicals as given in the list of ICH guidelines [2].

Isoniazid (INH) and pyrazinamide (PYZ) (Fig. 1) has been classified as the recommended first-line antitubercular drugs for prevention and treatment of tuberculosis in adults [3]. In tuberculosis, first line agents have to be taken for longer periods at a prescribed time interval and hence the quality of the product should be maintained for longer period. A safe, reliable and economical method has to be incorporated to find out the concentration of these drugs simultaneously as they are available in combined as well as single dosage.

Extended review reveals that various analytical methods based on amperometric [4], voltametric [5,6], spectrophotometric [7–13], spectrofluorometric [14], HPLC [15–23], HPTLC [24–26], RP-HPTLC [27] determinations are reported for identification and estimation of these drugs in fixed dosage form. The International Pharmacopoeia [28] has reported the simultaneous determination of INH and PYZ using RP-HPLC. So with this in view, this paper describes the method on supercritical fluid chromatography (SFC) and mass spectrometry for simultaneous determination of INH and PYZ and also to check if any impurity is present in the API as well as the interference from the excipient present in the fixed dose combination (FDC).

2. Experimental

2.1. Instruments

2.1.1. Supercritical fluid chromatograph

A JASCO-2000 series (Japan Spectroscopic Co. Ltd., Hachioji, Japan) of supercritical fluid chromatograph was used for the separations in this study. It was equipped with two pumps (PU-2080 and PU-2080 CO₂), which were capable to adjust the flow rate (0.001 to 10 ml/min.) for both Supercritical CO₂ and modifier. The system pressure was maintained electronically by back-pressure regulator (BP-2070), which allowed the flow rate and pressure to be controlled independently. An external loop with a capacity of 20 µl was equipped with rheodyne injector, capable to inject liquid sample accurately into the analytical column. The temperature of the column was thermostatically controlled in a column oven.
was detected by using a UV detector (Jasco-UV-2070). The effluent coming from the SFC was detected in the MS/MS for detection of any impurity if present.

2.1.2. Mass spectrometer

An AB Sciex (Canada) QTRAP-4500 series mass spectrometer was used in the present investigation. It was equipped with exclusive TurboV™ source contained TurbolonSpray probes which provide advanced linear ion trap technology for the highest level of sensitivity. The TurboV ion chamber has embedded ceramic heater technology with improved gas dynamics. Data acquisition and integration were done by Windows-based analyst software.

2.1.3. SFC/MS/MS conditions

Analysis was performed on fused silica column (inertisil ODS-5 μm C18, 150 mm × 4.6 mm) protected by precolumn filter cartridges. After optimization, mobile phase consisting of dichloromethane:methanol:formic acid (50:50:0.1 v/v/v) was used at flow rate of 0.3 ml/min and Supercritical CO₂ was flowed at 2 ml/min.

The optimized value for MS/MS analyses were as follows: ESI positive ion mode; capillary voltage, 3.5 kV; cone voltage, 40 V; Gas 1 (nebulizing gas) and Gas 2 (cone gas) were set to 50 units each and the source temperature was set at 550°C. High-purity nitrogen was used as nebulizer and cone gas.

The injection volume and column temperature were set at 20 μl and 40 °C, respectively. Full-scan SFC–MS/MS spectra were obtained by scanning from m/z 50 to 500.

2.2. Materials

Isoniazid and pyrazinamide standard was obtained as endowment samples from Sunij Pharma Pvt. Ltd. (Vatva GIDC, Ahmedabad) and tablet containing both the drug were procured from local market. Dichloromethane (HPLC Grade) and Methanol (HPLC Grade)—Lichrosolv®—was purchased from E. Merck (India) Ltd., Mumbai. Whatman filter paper no. 42 (0.45 μm) was used to filter the solutions.

2.3. Method

2.3.1. Selection of analytical wavelength

The overlay spectra of INH and PYZ were taken and found that at λ_max 262 nm, both the drug exhibit linear correlation and can be detected at the nano-gram level. So 262 nm was chosen as detection wavelength in SFC.

2.3.2. Preparation of mobile phase

A blend of 50 ml Methanol, 50 ml of Dichloromethane and 0.1 ml Formic acid was filtered through 0.45 μm filter paper. After filtration, the blend was sonicated for 10 min to degas the mixture and used as mobile phase.

2.3.3. Preparation of INH and PYZ stock standard and working solutions

The INH and PYZ stock standard was prepared by dissolving 10 mg of INH and 25 mg of PYZ in 10 ml mobile phase containing dichloromethane:methanol:formic acid (50:50:0.1 v/v/v). This solution was kept in refrigerator at 2–8 °C. The working solutions were obtained by suitably diluting the INH and PYZ stock solution.

2.3.4. Preparation of calibration standards and quality control (QC) samples

The appropriate volume of aliquots from standard INH and PYZ was transferred to volumetric flasks of 10 ml capacity to prepare six calibration standards. The volume was adjusted to the mark with mobile phase giving a solution containing 1–6 μg/ml for INH and 2.5, 5, 7.5, 10, 12.5 and 15 μg/ml for PYZ.

2.3.5. Determination of INH and PYZ from dosage form

For the analysis of dosage forms, not less than 20 tablets were weighed and make uniform powder. To prepare assay sample solution, weigh powder equivalent to 1 and 5 mg of INH and PYZ, respectively, was transferred to a clean and dry 10 ml of volumetric flask containing 5 ml of mobile phase as diluting solution and shaken thoroughly to extract the drug from the excipients and then sonicated for 10 min for complete dissolution of the drug (standard addition can be done to make sure the complete integrity of final concentration). The solution was allowed to cool at room temperature and then the volume was made up to the mark with the same diluting solution. The solution was filtered through Whatman filter paper (no. 42) and sonicated for 10 min. The appropriate volume of this prepared solution was taken and transferred to the volumetric flask of 10 ml capacity and volume was made up to the mark with the mobile phase to give a solution containing 3 μg/ml and 7.5 μg/ml INH and PYZ, respectively. This solution was used for the estimation of INH and PYZ (Table 2).

2.4. Method validation

Method validation was performed by using international guidelines [29,30] for determining selectivity, limits of quantification (LOQ) and detection (LOD), linearity and recovery. To assess the selectivity of the proposed method, spiked and non-spiked samples were injected into the SFC/MS system. The detection (LOD) and quantification limits (LOQ) were experimentally determined by injecting a number of non-spiked samples (n = 6) and measuring the magnitude of the background analytical response. The LOD and LOQ were estimated as three and ten times the signal-to-noise (S/N) ratio, respectively. The recovery was determined in 6 replicates at 3 concentrations (low, medium and high QC levels).

3. Results and discussion

The goal of this work was to provide an ecofriendly selective alternate method for determination of the INH and PYZ in FDC by SFC–MS/MS. In earlier work, we found that the addition of a low level of a volatile acid, such as formic acid, to the mobile-phase modifier make a dramatic improvement in the elution of polar and ionic molecules by SFC–MS/MS [31,32]. We therefore added 1 mM formic acid to the methanol:dichloromethane (50:50 v/v) as a modifier in the SFC/MS work.

3.1. Selection of mobile phase

For the selection of mobile phase, we have varied the concentration of modifier Methanol and Dichloromethane with the addition of 0.1% of formic acid ranging from 30% to 100% at a flow rates
Table 1
Regression analysis data of calibration curves prepared by the proposed method.

<table>
<thead>
<tr>
<th>Concentration of INH and PYZ (µg/ml)</th>
<th>Area INH Mean ± S.D. (n = 6)</th>
<th>(%RSD)</th>
<th>Area PYZ Mean ± S.D. (n = 6)</th>
<th>(%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 2.5</td>
<td>298,614 ± 768</td>
<td>0.26</td>
<td>390,347 ± 449</td>
<td>0.11</td>
</tr>
<tr>
<td>(2) 5</td>
<td>542,435 ± 1217</td>
<td>0.22</td>
<td>692,194 ± 1165</td>
<td>0.17</td>
</tr>
<tr>
<td>(3) 7.5</td>
<td>734,623 ± 714</td>
<td>0.10</td>
<td>947,274 ± 1092</td>
<td>0.12</td>
</tr>
<tr>
<td>(4) 10</td>
<td>936,962 ± 1673</td>
<td>0.18</td>
<td>1172,508 ± 1521</td>
<td>0.13</td>
</tr>
<tr>
<td>(5) 12.5</td>
<td>1126,940 ± 649</td>
<td>0.06</td>
<td>1417,901 ± 1252</td>
<td>0.09</td>
</tr>
<tr>
<td>(6) 15</td>
<td>1296,514 ± 2031</td>
<td>0.16</td>
<td>1689,852 ± 3149</td>
<td>0.19</td>
</tr>
</tbody>
</table>

ranging from 0.1 to 0.3 ml/min and supercritical carbon dioxide (SC-CO₂) with flow rates from 1.5 to 2.0 ml/min and chromatograms were recorded.

Amongst the all result obtained, the optimized system containing dichloromethane:methanol:formic acid (50:50:0.1 v/v/v) at 0.3 ml/min and CO₂ at 2 ml/min, was found to be satisfactory and gave well separate peak for INH and PYZ mixture (Fig. 2). Calibration data for INH and PYZ mixture is shown in Table 1. The calibration curve for INH and PYZ were prepared by plotting area and concentration (Fig. 3).

The following equations for straight line were obtained for INH and PYZ:

Linear equation for INH : \( y = 198,439x + 128,146 \) with regression coefficient \( R^2 = 0.9971 \).

Linear equation for PYZ : \( y = 101,713x + 161,692 \) with regression coefficient \( R^2 = 0.9983 \).

3.2. SFC–MS/MS optimization

The positive ionization mode in ESI was selected for ion production due to the presence of an amine group in the structures of INH and PYZ. Under UV trace no any other significant peak is observed in SFC, and as no any change in mass spectra of standard drugs observed, so we can ensure about the purity of the standard drug for further use in identification of these drugs in FDC. After collision-induced dissociation, the most abundant and stable product ions were at m/z 138.1 for INH and at m/z 124.1 for the PYZ (Fig. 4). Thus, the MRM transitions of m/z 160 → 100 and m/z 130 → 60 were selected for INH and PYZ, respectively. The mono-isotopic masses of INH and PYZ are 137.1 and 123.1, respectively. As a result, the masses of their protonated molecular ions were found to be 138.1 and 124.1 for INH and PYZ, respectively. After the analysis of these drugs in FDC, when compared with mass spectrum of standard drug it was observed that there is no any interference observed from the excipient. So, we can routinely use this method for determination of INH and PYZ in FDC.

3.3. Application of method

The validated method was successfully applied to determine the content of INH and PYZ in three different FDC. All the samples were analyzed in triplicate and percentage of content was determined and found to be lies within the limit (Table 2). Method validation has been discussed in following section.

![Fig. 2. Chromatogram of standard INH and PYZ in proposed mobile phase.](image)

![Fig. 3. Calibration curve of (a) INH and (b) PYZ in proposed mobile phase.](image)
3.4. Method validation

The developed SFC–MS/MS method was validated as per international guidelines [29,30].

3.4.1. Accuracy

To investigate the accuracy in sample preparation (i.e., extraction efficiency), we prepare a spiked solution by adding known amounts of related substances into a sample matrix. Thereafter responses of the spike solutions and the neat standard solutions were taken to assess the recovery from the sample preparation. At this stage, 20 tablets were taken and analysis of the sample was carried out. Recovery studies were carried out by the addition of standard drug to the sample at 3 different concentration levels (80%, 100%, 120%) taking into consideration percentage purity of added bulk drug samples. The method was found to be accurate with % recovery 98.89%–100.33% for INH and 99.27%–100.06% for PYZ (Table 3).

3.4.2. Precision

Precision was calculated as repeatability (Table 4) and intra- and interday variation for both INH and PYZ. The method was found to be precise with %RSD 0.10–0.20 for intraday (n = 3) and %RSD 0.11–0.21 for interday (n = 3) for INH and %RSD 0.13–0.16 for intraday (n = 3) and %RSD 0.13–0.16 for interday (n = 3) for PYZ (Table 5).

3.4.3. Robustness

Robustness of the method is determined by two operators (2 and 3) other than operator writing this paper, using standard method as described in this paper under different chromatographic conditions than those used in the present method. The chromatographic conditions and the results obtained are listed in Table 6.

Table 2
Analysis of marketed formulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>INH (mg)</th>
<th>PYZ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.D. (n = 3)</td>
<td>% RSD</td>
<td>Mean ± S.D. (n = 3)</td>
</tr>
<tr>
<td>FORECOX, Macleods Pharma (INH 100 mg, PYZ 500 mg)</td>
<td>98.53 ± 0.51</td>
<td>0.52</td>
</tr>
<tr>
<td>RF-3, Sunij Pharma (INH 100 mg, PYZ 500 mg)</td>
<td>99.64 ± 0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>R-CINEX-EZ, Lupin Lab. (INH 150 mg, PYZ 750 mg)</td>
<td>148.32 ± 1.25</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 3
Results of recovery study of INH and PYZ.

<table>
<thead>
<tr>
<th>Amount of drug</th>
<th>Amount of drug added</th>
<th>Total amount recovered</th>
<th>(% Recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH (µg/ml)</td>
<td>PYZ (µg/ml)</td>
<td>INH Mean ± S.D. (%)</td>
<td>INH Mean ± S.D. (%)</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>2.4</td>
<td>6.53 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3</td>
<td>6.02 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>7.5</td>
<td>13.43 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>7.5</td>
<td>5.34 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>3.6</td>
<td>9</td>
<td>6.53 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>7.5</td>
<td>13.43 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>3.6</td>
<td>9</td>
<td>6.53 ± 0.06</td>
</tr>
</tbody>
</table>

Table 4
Repeatability data for INH and PYZ.

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Conc. (µg/ml)</th>
<th>Area Mean ± S.D. (n = 3)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>3</td>
<td>734.58 ± 589</td>
<td>0.08</td>
</tr>
<tr>
<td>PYZ</td>
<td>7.5</td>
<td>947.04 ± 1129</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Fig. 4. MS/MS spectrum of (a) INH and (b) PYZ.
Table 5
Precision data for INH and PYZ.

<table>
<thead>
<tr>
<th>Name of drug</th>
<th>Conc. (µg/ml)</th>
<th>Intraday precision</th>
<th>Interday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Area Mean ± S.D. (n = 3) (%)</td>
<td>RSD</td>
</tr>
<tr>
<td>INH</td>
<td>2</td>
<td>542,721 ± 1069</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>734,126 ± 725</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>936,782 ± 1476</td>
<td>0.16</td>
</tr>
<tr>
<td>PYZ</td>
<td>5</td>
<td>692,418 ± 978</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>947,512 ± 1599</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1172,364 ± 1491</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 6
Robustness for INH and PYZ.

<table>
<thead>
<tr>
<th>Operator</th>
<th>Pharmaceutical</th>
<th>INH (mg)</th>
<th>PYZ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D. (n = 3) (%)</td>
<td>RSD</td>
</tr>
<tr>
<td>1</td>
<td>Tablet</td>
<td>99.15 ± 0.59</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>Tablet</td>
<td>98.63 ± 0.54</td>
<td>0.55</td>
</tr>
<tr>
<td>3</td>
<td>Tablet</td>
<td>100.07 ± 0.89</td>
<td>0.89</td>
</tr>
</tbody>
</table>

1 Conditions: [mobile phase—methanol: dichloromethane (40:60 v/v), flow-rate 0.2 ml min⁻¹, SC- CO₂ 2 ml min⁻¹ column temperature 38°C and UV detection, at 257 nm].

* Conditions: [mobile phase—methanol: dichloromethane (60:40 v/v), flow-rate 0.4 ml min⁻¹, SC- CO₂ 2.5 ml min⁻¹ column temperature 35°C and UV detection, at 267 nm].

Table 7
Summary of validation parameter for INH and PYZ.

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>INH</th>
<th>PYZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (µg/ml)</td>
<td>1–6</td>
<td>2.5–15</td>
</tr>
<tr>
<td>Slope</td>
<td>198,439</td>
<td>101,713</td>
</tr>
<tr>
<td>Intercept</td>
<td>128,146</td>
<td>161,692</td>
</tr>
<tr>
<td>R²</td>
<td>0.9971</td>
<td>0.9983</td>
</tr>
<tr>
<td>% Recovery</td>
<td>98.89–100.33</td>
<td>99.27–100.06</td>
</tr>
<tr>
<td>Precision (%)</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>(RSD)</td>
<td>0.04–0.20</td>
<td>0.13–0.16</td>
</tr>
<tr>
<td>Repeatability (n = 6)</td>
<td>0.10–0.20</td>
<td>0.13–0.16</td>
</tr>
<tr>
<td>Interday (n = 3)</td>
<td>0.11–0.21</td>
<td>0.13–0.16</td>
</tr>
<tr>
<td>Robustness (RSD)</td>
<td>0.55–0.89</td>
<td>0.46–1.14</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>11.87</td>
<td>35.42</td>
</tr>
<tr>
<td>LOQ (ng/ml)</td>
<td>35.97</td>
<td>107.36</td>
</tr>
</tbody>
</table>

parameters is tabulated in Table 6. Final optimized conditions are tabulated in Table 7.

4. Conclusion

To the best of our knowledge this is the first method that uses the principle of green chemistry with the support of tandem mass spectroscopy to determine isoniazid and pyrazinamide in fixed dose combination. The method has been successfully applied on three pharmaceutical dosage forms of different manufacturer. The reported method offers the advantage of the usage of ecofriendly method which separates both the component within the 5 min without any interference from the excipient.

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